ICH Guidance in Practice: Validated Reversed-Phase HPLC Method for the Determination of Active Mangiferin from Extracts of *Mangifera indica* Linn

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Abstract

This study presents the development and validation of a reversed-phase liquid chromatographic method for the determination of mangiferin (MGN) in alcoholic extracts of *mangifera indica*. A Lichrospher 100 C_{18}–ODS (250 × 4.6 mm, 5 µm size) (Merck, Whitehouse Station, NJ) prepacked column and a mobile phase of potassium dihydrogen orthophosphate (0.01M) pH 2.7 ± 0.2–acetonitrile (15:85, v/v) with the flow rate of 1 mL/min was used. MGN detection was achieved at a wavelength monitored at 254 nm with SPD-M 10A vp PDA detector or SPD 10AD vp UV detector in combination with class LC 10A software. The proposed method was validated as prescribed by International Conference on Harmonization (ICH) with respect to linearity, specificity, accuracy, precision, stability, and quantification. The method validation was realized using alcoholic extracts and raw materials of leaves and barks. All the validation parameters were within the acceptable limits, and the developed analytical method can successfully be applied for MGN determination.

Introduction

Mangiferin (1,3,6,7 tetra hydroxyl xanthone- C2- beta-D-glucoside, MGN) (Figure 1) was obtained from the dried stem bark and leaves of *Mangifera indica* Linn. This tree belongs to the family Anacardiaceae, which can be found wild or cultivated throughout the country. MGN could be a useful therapeutic compound in therapies for degenerative diseases, including Parkinson's disease, in which oxidative stress plays a crucial role (1). MGN was also reported to exhibit antioxidant (2), antiviral (3), cardioprotective and hypolipidemic (4,5), neuroprotectant (6), radioprotection (7, 8), anticryptosporidial (9), anti-inflammatory (10), diuretic, chloretic activities, and immunopathological disorders (11), including bronchial asthma, atopic dermatitis, and other allergic diseases (12). There have been several reports for MGN determination or determination in combination with other ingredients, including capillary electrophoresis (13), liquid chromatography with tandem mass spectrometry (LC–MS–MS) (14), reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection in Chinese preparation (15), in rat plasma and urine (16), in honey bush tea (Zhimu decoction) (17), using photo-colorimetric (18), spectrophotometric (19), and spectrofluorimetric method (20). Literature reveals that no analytical methods are available for MGN determination in leaves and bark extracts of mango tree. Method validation is the most important part of the analysis, and that is why there is a need to validate the developed method for the MGN determination in alcoholic extracts and raw materials of *mangifera indica*.

Experimental

Material and reagents

MGN reference standard (batch no. T2C036 certified to be 96.5% purity), three alcoholic extracts (MI-O5 lot 1, MI-O6 lot 1, and PC/MI/2610) and raw material (leaves and barks, ACD-497 and ACD-062, respectively) were kindly donated from Natural Remedies Pvt. Ltd. (Bangalore, India). The solvents acetonitrile and methanol from Qualigenes (Srinidhi Scientifics, Bengalooru, India), dimethyl formamide (DMF), and orthophosphoric acid from Rankem (RFCL Limited, Bengalooru, India) of HPLC-grade, potassium dihydrogen orthophosphate from S.D. Fine-Chem (Ashiwini Enterprises, Bengalooru, India) of analytical reagent-grade were purchased and used. All the solutions were used after filtration through Ultipor N_{66} Nylon 66 membrane (0.45 µm) P/N 60172 filter (Pall Life Sciences, Mumbai, India), and water of ultrapure-grade of 18 MΩ-cm resistance was obtained by a Arium 611 UV purifier (Sartorius Mechanotrics, Bengalooru, India).

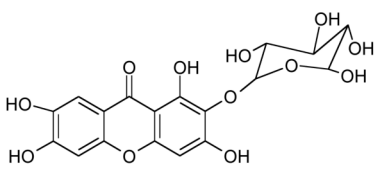

Figure 1. Chemical structure of mangiferin.

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Apparatus and software

The Shimadzu HPLC-LC2010A was equipped with isocratic pump, autosampler, and SPD-M 10A vp PDA detector or SPD 10AD vp UV detector (Kyoto, Japan). Data acquisition and peak integration analysis were performed using CFR – 21 part II software. Separation was achieved on a Lichrospher 100 C_{18}–ODS (octadecyl silane) (250 × 4.6 mm, 5 µm size) (Merck, Whitehouse Station, NJ) column. The column temperature was maintained at 27°C. Injection volume was 20 µL and UV detection at 254 nm. Total run time was 15 min. Analytical weighing balance Sartorius-BP-211D (Sartorius, Goettingen, Germany) was used as well as the DLF vacuum filter pump (DLF Universal Limited, Delhi, India).

Procedures

Preparation of buffer solution

The mobile phase was prepared by dissolving 1.36 g of potassium dihydrogen orthophosphate in 900 mL of water, and the pH was adjusted to 2.7 ± 0.2 using dilute orthophosphoric acid. Finally, the volume was made up to 1000 mL with water. This buffer solution was mixed with acetonitrile to obtain a final ratio of 15:85 (v/v). The mobile phase of 0.01 M potassium dihydrogen orthophosphate (pH 2.7 ± 0.2)–acetonitrile (15:85, v/v) was used with an isocratic mode at a flow rate of 1 mL/min.

Table I. Calibration Parameters of the Proposed HPLC Method for Determination of Mangiferin

Parameters	
Calibration range (µg/mL)	15–1000
Regression equation (Y)*	Y = a + bC
Slope (b)	44,574.000
Standard deviation of the slope (Sb)	267.640
Relative standard deviation of the slope (%)	10.616
Confidence limit of the slope†	104.223
Intercept (a)	496,760.667
Standard deviation of the intercept (Sa)	52,734.394
Relative standard deviation of the slope (%)	0.600
Confidence limit of the intercept†	20,535.661
Correlation coefficient (r)	0.9996
Response factor‡	48,969.1861

* where C is the concentration of compound in µg/mL and Y is the peak area.
† 95% Confidence limit, (n = 3). ‡ Response factor = peak area / Concentration.

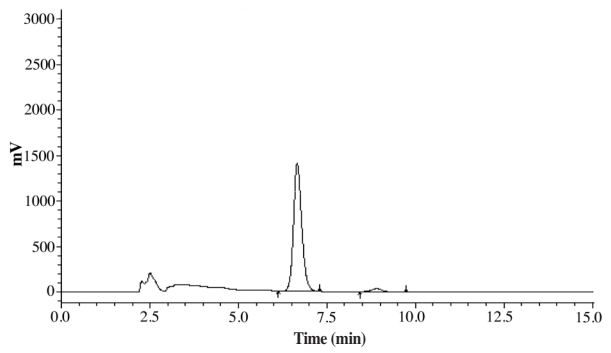

Figure 2. Chromatogram showing retention time of mangiferin.

Preparation of mangiferin calibration standards

In a 10-mL volumetric flask, a MGN stock solution of 973.589 µg/mL was prepared by dissolving 10.089 mg (96.5% purity) of the compound of interest with 1 mL of DMF and a small amount of HPLC methanol. Then this solution was sonicated for 5 min and warmed on a steam water bath for 5 min, cooled, and made up to 10 mL with HPLC methanol. Calibration standards at seven levels of concentrations were prepared by sequential dilutions of the stock solution with the HPLC methanol to get 973.589, 486.794, 243.397, 121.699, 60.849, 30.425, and 15.212 µg/mL for the analytical range 15–1000 µg/mL. Injections of 20 µL in triplicate were made from each concentration and chromatographed under the specified conditions described.

Preparation of samples

Sample solutions for extracts

For all the validation experiments the sample solutions from alcoholic extracts (MI-O5 lot 1, MI-O6 lot 1, and PC/MI/2610) were prepared by weighing different amounts (shown in respective tables) of each samples and dissolving separately in 10 mL of DMF, sonicated, and warmed on a water bath for 5 min each, cooled, and made up the volume to 100 mL with methanol.

Sample solutions for raw material

The sample solutions from raw material (coarse powder) were prepared by transferring the weighed amount (420.05 mg and 631.8 mg of ACD-497 in accuracy studies and quantification, respectively, and 839.3 mg of ACD-062 for quantification) to a 100-mL beaker. Extract with 10 mL of DMF and 80 mL of methanol sonicated for 5 min by warming on a water bath for about 20 min; discard the supernatant liquid extract to a 500-mL beaker. Repeat the procedure four more times until the raw material is completely extracted or until the extract is colorless. The extract was collected, mixed well, concentrated to less than 100 mL, and made up the volume to 100 mL with methanol, and filtered through 0.45-µm membrane filter.

Results and Discussions

To meet the current pharmaceutical ICH (21) regulatory guidelines, a number of parameters have been investigated in order to validate our analytical method such as linearity, speci-

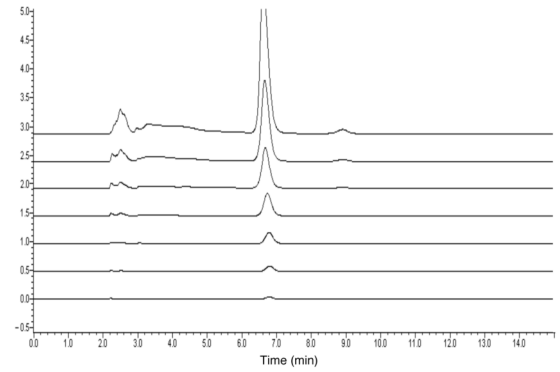

Figure 3. HPLC 2-dimensional chromatogram offset of linear concentrations set of mangiferin standard.

ficity, accuracy, stability, precision, range, and quantification. The reversed-phase HPLC method was developed to provide a specific procedure suitable for the rapid quality control analysis of MGN content in *mangiferin indica* linn. The method involves the use of an RP-C_{18} column, and a mobile phase consisted of potassium dihydrogen orthophosphate (0.01M) pH 2.7 ± 0.2–acetonitrile (15:85, v/v). This combination of the mobile phase was chosen after several trails with different solvents, ratio, and pH. A change of the wavelength was performed during the run in order to achieve the maximum detector response and the best chromatogram without interfering peaks. The chromatographic system described allows validating the method with reliable results.

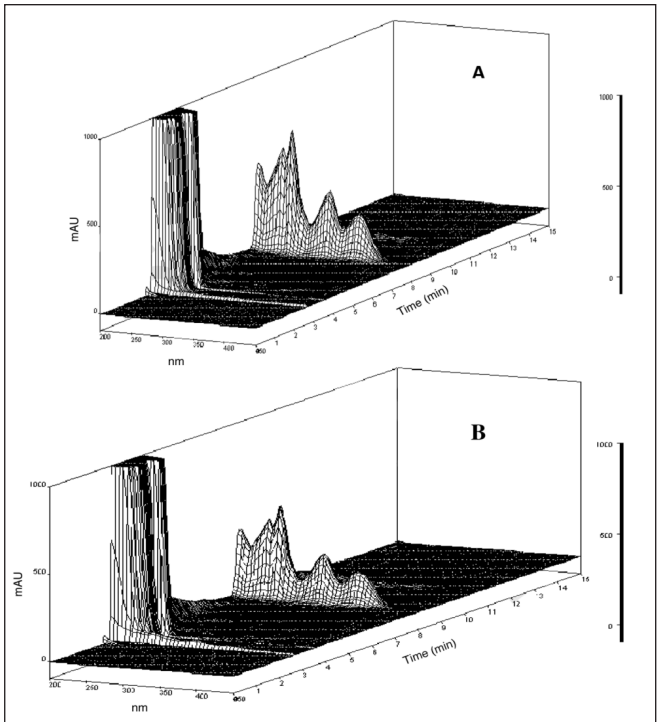

Figure 4. 3D- PDA spectrum of mangiferin (A) standard and (B) sample (MI-05 Lot1).

Method validation

Linearity of response

Linearity was evaluated across the range the analytical standards used. The nominal working concentration for MGN determination ranges between 15–1000 µg/mL. The linearity was performed over the range of 15.212–973.589 µg/mL. The calibration curve was obtained using the linear least squares regression procedure, and the representative linear equation parameters are shown in Table I. The coefficient of correlation, slope, intercept, and % relative standard deviation (RSD) are suitable as a general acceptable criterion to the linearity performance of an analytical procedure. The two-dimensional (2D) chromatogram offset representing the linearity of the method is shown in Figure 2.

Specificity (selectivity)

A representative three-dimensional peak purity spectrum, acquired by PDA detector using MGN standard and sample MI-O5 lot 1, are presented in Figures 3A and B. This demonstrates the high degree of selectivity and that the peak of interest is attributed only to MGN (peak purity 0.997, Figure 4). No endogenous interference was observed at the retention time of MGN (Figure 5). The PDA spectrum of MGN standard with that of the PC/MI/2610 and ACD/062 were also tested, and their 3D spectrum demonstrated the presence of the analyte in the samples, indicating peak purity greater than 98%.

Accuracy

Accuracy was determined by using our method and by spiking MGN indica raw material samples (ACD/062) and extracts (MI-O5 lot 1, MI-O6 lot 1) with known amounts of MGN standards and compared the measured value with the true values. Triplicates injections were made with all the spiked samples. Table II summarizes the accuracy results, expressed as a recovery percentage. The method has shown 96.41, 99.64, and 101.31% recovery of samples ACD/062, MI-O5 lot 1, and MI-O6 lot 1, respectively.

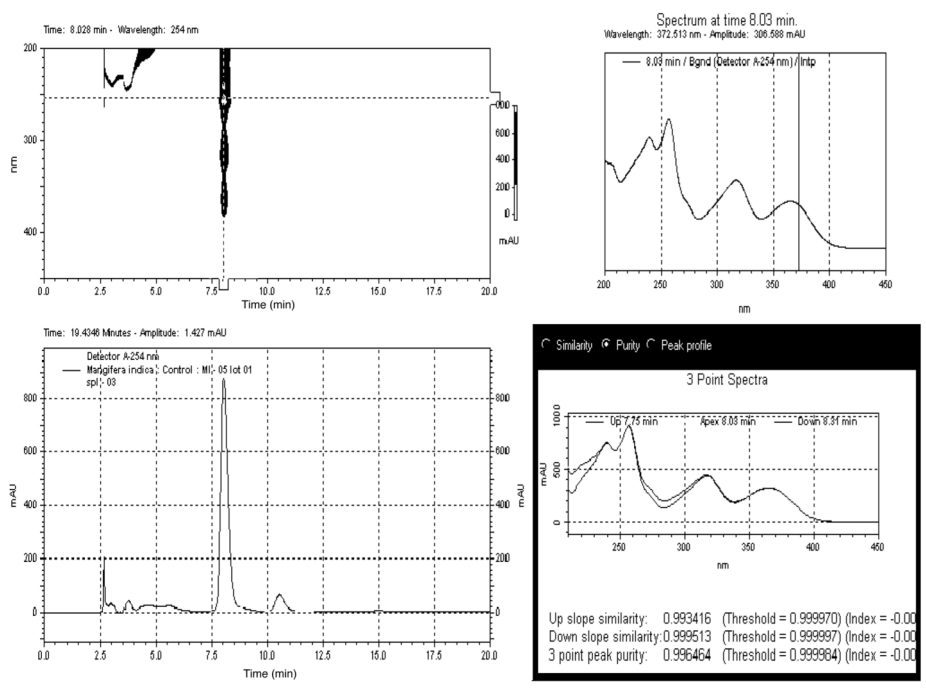

Figure 5. PDA spectrum of mangiferin sample (MI-05 Lot1) representing purity of spectra.

Precision

It was determined by performing triplicate analyses of the standards spiked with samples. Thus, repeatability was demonstrated by spiking standards with 12 different concentrations from sample (MI-O5 lot 1) stock solution and by comparing the % recovery with that of the reference standard solution of 126.699 µg/mL

Table II. Accuracy Studies Using Standard Addition Technique

Samples	MI-O5 lot 1	MI-O6 lot 1	ACD/062
Sample (mg)	102.56	81.2	420.5
Std added (mg) (96.5 % purity)	3.5	5.07	3.29
Actual wt. of std added (mg)	3.38	4.89	3.17
Total weight (mg)	106.06	86.27	423.79
Dilution (mL)	100.00	100.00	100.00
% Mangiferin content in the sample	18.45	21.4	8.23
Mangiferin present in the sample (mg)	18.92	17.38	34.61
Total Mangiferin (mg)	22.30	22.27	37.78
Theoretical value*	21.03 %	25.81 %	8.92 %
Standard wt (mg)	2.52	2.52	2.52
Standard dilution (mL)	10	10	10
Standard area†	11,506,455	11,506,455	11,506,455
Sample area†	10,173,127	10,499,525	18,111,404
Calculation‡	20.27 %	25.72 %	9.03 %
% Recovery	96.41	99.64	101.31

*Theoretical value = (Total Mangiferin / Total weight) × 100.
†Average of three trials.
‡Calculated value = (Sample area/Standard area) × (Standard weight / Standard diluation) × (Dilution/Total weight) × 96.5.

Table III. Precision Studies Performed for Determining Mangiferin Using (MI-O5 lot 1) Sample

Sl. No	Wt. of sample (mg)	Peak area*	Mangiferin found (% w/w)
1	27.54	2,674,473	18.40
2	32.68	3,158,349	18.32
3	50.45	4,890,591	18.37
4	60.46	5,838,719	18.30
5	110.68	10,749,602	18.41
6	109.21	10,757,648	18.67
7	214.56	20,976,761	18.53
8	210.65	20,344,254	18.30
9	368.29	35,398,650	18.22
10	403.57	39,788,973	18.69
11	450.59	44,241,633	18.61
12	450.65	44,162,574	18.57
Mean			18.45
Std. Dev			0.16
% RSD			0.86

*Average of three experiments.

Table IV. Robustness of Chromatographic Method

Parameter	Peak asymmetry
Flow rate	
0.8	1.379 ± 0.061
1.0	1.224 ± 0.03
1.2	1.243 ± 0.033
Acetonitrile % in mobile phase	
13	1.25 ± 0.001
15	1.224 ± 0.03
17	1.267 ± 0.002
Change in pH	
2.4	1.279 ± 0.00
2.7 ± 0.2	1.224 ± 0.03
3.0	1.366 ± 0.038

under the same condition. The % recovery, standard deviation, and % RSD are shown in Table III. The precision of the proposed method was good demonstrating % RSD less than 1.0%.

Robustness

The robustness of the proposed HPLC method was assessed by purposely altering the chromatographic conditions such as mobile phase flow rate (± 0.2), mobile phase organic content (± 2%), apparent pH of the mobile phase (± 0.3), and the parameter investigated for change in peak asymmetric factor (Table IV). The study did not have significant effect on peak symmetric factor and, hence, the determination of MGN.

Stability of the solutions

The stability of the freshly prepared MGN sample (MI-06 lot 01) and standard solutions with respect to the time was investigated. A quantity of 150.9 mg of sample extract was taken to prepare a concentration of 323.07 µg/mL solution. This sample solution was spiked with standard solution. Immediately, the standard and spiked sample solutions after preparation were injected onto the HPLC system at 0, 6, 12, and 24 h. The data obtained were evaluated with respect to peak area (Table V). No interfering or sample degradation peaks were observed at analyte retention time.

Quantification

The quantification (% purity) of the samples (both extracts and raw materials), which are used in validation process, was further realized by applying the developed method to ensure the correctness of their % purity. The results obtained are shown in Table VI. The same % purity for all samples was claimed throughout the validation process.

Table V. Stability Study Results Using Sample (MI–O6 lot 1)

Time (min)	Peak Area*	Result (% w/w)
0 h	16,987,846	21.34
6 h	17,017,545	21.37
12 h	17,058,999	21.42
24 h	17,055,367	21.42

*Average of three experiments.

Table VI. Quantification Data Revealed On Samples Against Reference Standard

Samples	Weight (mg)	Peak Area*	Standard deviation	RSD %	Mangiferin found (% w/w)
ACD-497	839.3	6,791,893	85,686.4	1.26	1.53
ACD-062	631.8	27,440,074	860,314.8	1.14	8.23
MI-05 lot 1	154.7	14,892,870	103,445.9	0.69	18.45
MI-O6 lot 1	106.7	12,054,201	119,008.4	0.99	21.41
PC/MI/2610	20.9	9,384,770	371,750.3	1.96	85.1

* Average of three experiments.

Conclusions

Here, we have developed and validated an optimized new reversed-phase liquid chromatographic method for the analysis of mangiferin extracted from *M. indica* Linn. The developed method yields validated results realized on different purity samples, demonstrating high degree of accuracy, specificity, and precision. Therefore, the developed RP-HPLC method was proved to be suitable for the mangiferin determination in various alcoholic extracts and raw materials of *M. indica* Linn in quality control laboratories.

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